PROTEIN SEPARATION DEVICE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S Provisional Patent Application Number US 60/530,608, which is incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to a device for the separation of proteins, in particular the separation of low molecular weight proteins from high molecular weight proteins, in a fluid sample, in particular a biological fluid sample; a method for using the device and to proteins obtainable by way of such a method.

BACKGROUND OF THE INVENTION

The study of the human proteome, in particular the human serum proteome, is an area of great interest, especially with respect to the pharmaceutical industry, with its potential to identify disease or biological markers. Studying this proteome presents a major challenge due to the varying concentrations of the constituent proteins of serum. These concentrations can vary by approximately ten orders of magnitude. Most of the pharmaceutically useful proteins are of the low molecular weight type and are found in low concentrations.

Human serum is typically comprised of blood with its constituent cells (erythrocytes and leucocytes) and clotting factors removed. The protein concentration of the serum is usually in the range of from 50 to 70mg/ml. Approximately 70% of this protein is serum albumin (30 to 35mg/ml) and 10% is IgG (5 to 7mg/ml).

There are at least 10,000 proteins in human serum but most, approximately 95%, are at very low concentrations and have low molecular weights. For example, interleukin 6 (a marker for inflammation and/or infection) has a molecular weight of 21kDa and is present in serum at a concentration of 10pg/ml; a concentration of almost ten orders of magnitude less than serum albumin.

One of the most popular methods for examining the proteome is to use two-dimensional electrophoresis (2DE). Typically, this involves the separation of proteins by their isoelectric point and then by their molecular weight by SDS-PAGE.

2DE is advantageous as it has the potential to separate several thousand proteins as spots on one gel. The spots can then be excised from the gel, digested with trypsin and identified using MALDI-MS (matrix-assisted laser desorption ionisation mass spectroscopy). Other methods used in the separation of proteins include high-performance liquid chromatography and SELDI-MS (surface-enhanced laser desorption ionisation mass spectroscopy).

However, these methods usually involve a process of prefractionation and can result in the non-specific removal of proteins of interest that are associated with other proteins that are not of interest.

SUMMARY OF THE INVENTION

The present invention provides a protein separation device comprising a chaperone protein immobilised on a substrate.

In another aspect, the present invention provides a protein separation device comprising GroEL immobilised on a substrate in an optimised orientation to bind a target

protein and to provide minimal steric hindrance between GroEL and the substrate.

In still a further aspect, the present invention provides a protein separation device comprising GroEL immobilised on a substrate, wherein the specificity of GroEL is directed to a particular protein.

In another aspect, the present invention provides a protein separation device comprising GroEL immobilised on a substrate, wherein the specificity of GroEL is changed to a specificity of another chperone protein.

In yet another aspect, the present invention provides a protein separation device comprising GroEL in an optimised orientation to bind a target protein and to provide minimal steric hindrance between GroEL and the substrate wherein the specificity of GroEL is directed to a particular target protein.

The present invention also provides, in another aspect, a protein separation device comprising GroEL immobilised on a substrate in optimised orientation to bind a target protein and to provide minimal steric hindrance between GroEL and the substrate wherein the specificity of GroEL is changed to a specificity of another chaperone protein.

The present invention also provides, in a further aspect, a method of isolating at least one protein from a biological sample comprising the steps of:

a) denaturing a biological sample containing at least one protein;

b) applying the biological sample containing at the least one protein to a chaperone protein immobilised on a substrate.

c) isolating the at least one protein from the biological fluid on the chaperone protein;

d) removing the biological sample from the chaperone protein immobilised on the substrate, and

e) obtaining the at least one protein from the chaperone protein.

The present invention also provides, in another aspect, A method of identifying a biological marker in a biological sample comprising the steps of:

a) applying the biological sample containing the biological marker to a chaperone protein immobilised on a substrate;

b) isolating the biological marker from the biological fluid on the chaperone protein;

c) removing the biological sample from the chaperone protein, and

d) obtaining the at least one protein from the chaperone protein immobilised on the substrate.

The present invention also provides, in another aspect, a method of diagnosis comprising the steps of:

a) applying a biological sample from a first subject to a chaperone protein immobilised on a substrate;

b) isolating a protein from the biological fluid on the chaperone protein;

c) removing the biological sample from the chaperone protein;

d) obtaining the at least one protein from the chaperone protein, and

e) Comparing the concentration of the at least one protein from the first subject with a reference concentration obtained from a second subject.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1a and 1b. Shows the peptide sequence and DNA codons of wild-type GroEL.

Figure 2a and 2b. Shows the peptide sequence and DNA codons of GroEL having Aspartate 490 substituted with Cysteine.

Figure 3a and 3b. Shows the peptide sequence and DNA codons of GroEL with the mutations Leucine 200→Arginine, Serine201→Tyrosine, Proline202→Aspartate and Aspatate490→Cysteine.

Figure 4a and 4b. Shows the peptide sequence and DNA codons of GroEL with the mutations Tyrosine199→Isoleucine, Tyrosine204→Isoleucine, Leucine234→Isoleucine, Leucine237→Isoleucine, Leucine259→Phenyalanine, Valine263→Leucine and Valine264→Phenylalanine and Aspartate490→Cysteine.

Figure 5. Shows a polyacrylamide gel of GroEL monomers of ~57kDa from eluted fractions of a column.

Figure 6. Shows a polyacrylamide gel of GroEL in a wild-type double heptamer ring configuration of ~840kDa from eluted fractions of a column.

Figure 7. Shows the results of an ATPase assay of biotinylated GroEL immobilised on NeutrAvidin beads.

Figure 8. Shows the results of a protein folding assay by biotinylated GroEL immobilised on NeutrAvidin beads.

Figure 9. Shows a polyacrylamide gel of purified GroES in eluted fractions from a column.

Figure 10. is an illustration of the mechanism of GroEL when immobilised on NeutrAvidin beads.

DETAILED DESCRIPTION OF THE INVENTION

MATERIALS

A biological sample may be obtained from a human subject. The sample is preferably a fluid but may also be some other biological extract. It will be appreciated that the application of the invention is not to be limited to humans but can be used on a biological sample from any animal. The fluid may comprise, but is not limited thereto, serum, cerebrospinal fluid, urine, nipple aspirant, other biological fluids, extracts, tissue extracts or other mixture of proteins.

CHAPERONE PROTEINS

Chaperone proteins bind to non-native (denatured) states of other proteins and assist them to reach a functional conformation. This is achieved in most cases through the expenditure of ATP. Originally identified by their increased abundance following heat shock, chaperone proteins in general recognise exposed hydrophobic surfaces of non-native species of proteins and form non-covalent interactions with them, stabilising them against

irreversible multimeric aggregation. Release of the polypeptide then follows, in many cases driven by an ATP-directed conformational change in the chaperone protein, permitting subsequent steps of polypeptide folding to occur. When such steps fail to proceed to a native conformation, recognition and rebinding by the same or other chaperone protein can occur, allowing another opportunity for a productive conformation to be reached.

Different classes of chaperones are directed to binding specific non-native states. For example, Hsp70 and Hsp60 (chaperonin) chaperones recognise, respectively, extended and collapsed conformations, which are bound correspondingly either by local enclosure of the polypeptide chain or by global enclosure of the polypeptide in a central enclosure.

The present invention takes advantage of the chaperone's ability to recognise a non-native state of a protein to separate that protein from other proteins present in a biological sample. Advantageously, chaperones are also able to select non-native proteins by their molecular weight. This feature of chaperones is also exploited to isolate low molecular weight proteins from a biological sample.

Advantageously the present invention will work with any chaperone protein.

The chaperone is preferably selected from the group consisting of Hsp100, Hsp90, Hsp70, Hsp60 and small Hsps, for example Hsp25 and the like. Preferably, the chaperone may be an Hsp60 chaperone of the group I chaperonin type. More preferably, the chaperone may be of the chaperonin type possessing peptide-dependent ATPase activity. Most preferably, the chaperonin may be, for example, GroEL.

Preferably, GroEL may be in operative association with a co-factor. The co-factor may be, for example, the co-chaperonin, GroES or a fragment thereof.

Although the mechanism of GroEL in protein folding is well documented in the art, it will be appreciated, by those of skill in the relevant art, that the following proposed mechanism is a theory and the invention should not be construed as being limited to any particular theory in the art or that proposed herein.

GroEL (also referred to as Hsp60) is of the group I chaperonin type. The term chaperonin refers to the double ring structure that these proteins generally comprise. Typically, these proteins present as heptameric, double-ring assemblies that promote the folding of proteins from a non-native or denatured state to the native state. The structure of GroEL is arranged in a back-to-back fashion of identical or closely related rotationally symmetrical subunits.

The rings of GroEL define a central, generally cylindrical, cavity that functions in two conditions. In a first operative condition, GroEL is open at an end of the cylindrical cavity to allow ingress of non-native proteins. The opening is provided with a flexible hydrophobic lining located in an apical domain of each subunit in the ring structure. The hydrophobic lining binds to non-native proteins in a multivalent interaction between their respective exposed hydrophobic surfaces.

In a second operative condition the binding of ATP, to an equatorial domain of GroEL, together with a co-chaperonin, GroES, at a location in an apical domain of GroEL *per se* induces a conformational change in the ring structure.

GroES is advantageous as it limits the size of proteins captured by GroEL to ~57kDa or less.

The conformational change preferably comprises the *en bloc* movement of the seven apical domains of each of the subunits in the ring structure resulting in a global change to the internal milieu of the central cylindrical cavity. The cavity increases in volume by almost two-fold and is closed off by GroES. The hydrophobic surface at the apical domain is then elevated and twisted away from the non-native polypeptide causing the peptide to be released into the central cavity. The cavity is now predominantly hydrophilic in character, favouring the burial of exposed hydrophobic amino acid residues and the promotion of the native state of the polypeptide.

The non-native protein may undergo many rounds of capture and release by GroEL until the polypeptide has refolded into its native state. The capture and release may be performed by the same GroEL protein or by different a GroEL protein.

Preferably, GroEL is used in its wild-type double ring structure of a chaperonin. Alternatively, GroEL may be utilised in a single, heptameric, ring form.

One ring of GroEL may comprise a heptameric ring and the other ring may comprise a dimeric, trimeric, tetrameric, pentameric or a hexameric structure.

GroEL may also, for example, comprise a heptameric ring of wild-type GroEL and a ring of another chaperonin protein, for example, rubisco subunit binding protein or CCT.

GroEL may further comprise a double ring assembly wherein one or both rings comprise one or more subunits from other chaperonins and each ring may be a heteromeric heptamer.

For example the rings may contain one or more of each of the α, β, γ, δ, ε, ζ or θ subunits of CCT (TCP-1) or one or more subunits of rubisco subunit binding protein. Alternatively, GroEL may comprise a double ring assembly wherein the or each ring comprises one or more subunits from other chaperonins.

GroEL used in the present invention may take any of the forms listed above, including fragments of chaperone proteins, and any combination thereof.

The chaperonin GroEL may be obtained from a microbial source selected from the group consisting of bacteria and archaea, for example, those of *Escherichia spp.*, *Thermus spp.* *Streptococcus spp.*, *Staphylococcus spp.*, *Bacillus spp.*, *Leptospira spp.*, *Spirillum spp.*, *Lactobacillus spp.*, *Mycoplasma spp.*, *Pseudomonas spp.*, *Streptomyces spp.*, *Corynebacterium spp.*, *Bacteroides spp.* and *Clostridium spp.*. GroEL is preferably isolated from *Escherichia coli*. Alternatively GroEL may be isolated from *Thermus thermophilus* or *Clostridium difficile*.

SUBSTRATE

The substrate is preferably a solid support of the array or bead type. These may be manufactured from any suitable material known to those of skill in the art, for example a plastics material. Typically, supports of the array type may be provided with a variety of surfaces, located in spots on the substrate, to permit the protein of the chaperone type to be immobilised thereon. These surfaces may be comprised of moieties selected from the group consisting of, *inter alia*, nitriloacetic acid, carboxylates, quaternary amines, silicates, carbonyl diimidazoles and epoxides. The

substrate may be provided with an hydrophobic barrier coating.

Suitable substrates for use in the present invention are, for example, bio-chips available from Ciphergen® or NeutrAvidin beads available from Pierce.

MODIFICATION OF THE CHAPERONE PROTEIN

The chaperone protein of the present invention may be modified in order to alter its properties. For example, the chaperone may be modified to improve its binding to a target protein or improve folding functions. Typically such modifications are achieved by deleting, introducing or mutating specific codons in the DNA/cDNA sequence of the chaperone. Typically this may be carried out using site-directed mutagenesis. Site-directed mutagenesis may be performed by polymerase chain reaction or some other suitable method known to those of skill in the art.

It will be appreciated that modifications made to the amino acid sequence of GroEL may be of the conservative type, for example, substitution of polar-to-polar, non-polar to non-polar or aromatic-to-aromatic residues. Alternatively non-conservative substitutions may be made to the amino acid sequence of GroEL. For example, polar to non-polar residue substitutions.

The modified amino acid sequence of GroEL may be 70-80% homologous to SEQ ID No. 2. The sequence may be 90-95% homologous to SEQ ID No. 2. Alternatively, the amino acid sequence may be 96, 97, 98 or 99% homologous to SEQ ID No. 2.

Homology/hybridization

"Homology" and "homologous" refers to sequence similarity between two peptides or two nucleic acid molecules. Homology can be determined by comparing each position in the aligned sequences. A degree of homology between nucleic acid or between amino acid sequences is a function of the number of identical or matching nucleotides or amino acids at positions shared by the sequences. As the term is used herein, a nucleic acid sequence is "homologous" to another sequence if the two sequences are substantially identical and the functional activity of the sequences is conserved (as used herein, the term 'homologous' does not infer evolutionary relatedness). Two nucleic acid sequences are considered substantially identical if, when optimally aligned (with gaps permitted), they share at least about 50% sequence similarity or identity, or if the sequences share defined functional motifs. In alternative embodiments, sequence similarity in optimally aligned substantially identical sequences may be at least 60%, 70%, 75%, 80%, 85%, 90% or 95%. As used herein, a given percentage of homology between sequences denotes the degree of sequence identity in optimally aligned sequences.

Substantially complementary nucleic acids are nucleic acids in which the complement of one molecule is substantially identical to the other molecule. Two nucleic acid or protein sequences are considered substantially identical if, when optimally aligned, they share at least about 70% sequence identity. In alternative embodiments, sequence identity may for example be at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%. Optimal alignment of sequences for comparisons of identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith

and Waterman, 1981, *Adv. Appl. Math* 2: 482, the homology alignment algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443, the search for similarity method of Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85: 2444, and the computerised implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence identity may also be determined using the BLAST algorithm, described in Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-10 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information (through the internet at http://www.ncbi.nlm.nih.gov/). The BLAST algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold. Initial neighbourhood word hits act as seeds for initiating searches to find longer HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when the following parameters are met: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program may use as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992, *Proc. Natl. Acad. Sci. USA* 89: 10915-10919)

alignments (B) of 50, expectation (E) of 10 (or 1 or 0.1 or 0.01 or 0.001 or 0.0001), M=5, N=4, and a comparison of both strands. One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In alternative embodiments of the invention, nucleotide or amino acid sequences are considered substantially identical if the smallest sum probability in a comparison of the test sequences is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

An alternative indication that two nucleic acid sequences are substantially complementary is that the two sequences hybridize to each other under moderately stringent, or preferably stringent, conditions. Hybridisation to filter-bound sequences under moderately stringent conditions may, for example, be performed in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C (see Ausubel, *et al.* (eds), 1989, *Current Protocols in Molecular Biology*, Vol. 1, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3). Alternatively, hybridization to filter-bound sequences under stringent conditions may, for example, be performed in 0.5 M $NaHPO_4$, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.1 x SSC/0.1% SDS at 68°C (see Ausubel, *et al.* (eds), 1989, *supra*). Hybridization conditions may be modified in accordance with known methods depending on the sequence of interest (see Tijssen, 1993, *Laboratory Techniques in Biochemistry and Molecular Biology -- Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of

nucleic acid probe assays", Elsevier, New York). Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point for the specific sequence at a defined ionic strength and pH.

As defined herein, the expression "GroEL" includes variants of native GroEL polypeptide, for example: deletions, including truncations and fragments; insertions and additions, including tagged polypeptides and fusion proteins; substitutions, for example site-directed mutants and allelic variants.

As used herein, "polypeptide" means any chain of amino acids, regardless of length or post-translational modification (*e.g.*, glycosylation or phosphorylation), and include: natural proteins; synthetic or recombinant polypeptides and peptides as well as hybrid molecules (e.g. a fusion protein or chimera having one portion comprising all or part of a polypeptide of the invention and a second portion comprising an amino acid sequence from another protein or peptide); modified peptides, including for example peptoids having one or more non-amino acyl groups (q.v., sugar, lipid, etc.) covalently linked to the peptide; and peptidomimetics. Typically the protein or polypeptide may be isolated or substantially pure or recombinant.

The modifications may include, without limitation, the introduction of a proteolytic cleavage site; an N- or O-linked glycosylation site in which the N-linked glycosylation site is a high mannose type, a hybrid type or a complex type glycosylation site; an acylation site, for example, a myristoylation site; a methylation site; a phosphorylation site; a sulphation site and a prenylation site, for example, a farnesyl or geranyl site.

Preferably, modification of the chaperone protein may be performed to:

a) optimise the orientation of the immobilised chaperone and minimise steric hindrance between the chaperone and a solid substrate;

b) direct the target specificity of the chaperone, and

c) alter the target specificity of the chaperone.

In order to optimise the orientation of the GroEL chaperonin and minimise steric hindrance, Asp490 may be substituted with a Cys residue (SEQ ID No. 2). This mutation introduces a thiol group in the equatorial domain of GroEL. The thiol group may, for example facilitate the introduction of biotin at this site. This is advantageous as immobilisation of GroEL on an array or a bead can be achieved by taking advantage of the interaction between biotin located in GroEL and a streptavidin moiety located on an array or a bead.

To direct the target specificity of the GroEL chaperonin, the mutations Leu200→Arg, Ser201→Gly and Pro202→Asp (SEQ ID No. 3) introduce, into the apical domain of the subunits of GroEL, a consensus binding motif, RGD, specific for the integrin family of proteins. Integrins are important in cell-to-cell and cell-matrix interactions; and have been implicated in cell signalling. The above mutations may also comprise, for example, the binding motifs RCD and RYD, which are also recognised by integrins. This is advantageous as modification of GroEL in the apical domain allows the target specificity to be directed towards a specific group or family of proteins.

It will be appreciated that any binding motif could be introduced into a binding domain of any chaperone used in the present invention in order to isolate a specific protein or family of proteins.

To alter the target specificity of GroEL, the mutations Tyr199→Ile, Tyr204→Ile, Leu234→Ile, Leu237→Ile, Leu259→Phe, Val263→Leu and Val264→Phe (SEQ ID No. 4) may be made in the apical protein-binding domain of GroEL. These mutations result in the replacement of the substrate binding specificity of GroEL, a group I chaperonin, with that of Thermosome, a group II chaperonin. This is advantageous as it allows the capture by the modified GroEL of targets that were previously unavailable to wild-type GroEL. Alternatively, other mutations may be made to replace the substrate binding specificity of GroEL with CCT or rubisco subunit binding protein.

This is advantageous as it allows for the possibility of increased capture of proteins by various forms of GroEL on an array or a bead. The array or bead may comprise one or more different types of mutated GroEL. Alternatively, for example, a first array or a bead may comprise only wild-type of GroEL and may be assayed with a biological sample in parallel with a second array or bead comprising a further type of mutated GroEL.

PREPARATION OF THE PROTEIN SEPARATION DEVICE

The protein separation device in accordance with the present invention may conveniently take the form of an array or a bead or other suitable solid support known to those skilled in the art.

The array or bead is preferably prepared in accordance with the manufacturer's instructions. Typically, for an array this involves the steps of:

a) rehydrating one or more spots located on the array in the recommended buffer;

b) loading the one or more spots with a chaperone, and

c) Incubating the array overnight in an humidifier.

Preferably, 1 to 10µg/ml of GroEL may be loaded on to the or each spot. More preferably 2 to 8µg/ml, more preferably 4 to 8µg/ml, more preferably 5 to 7µg/ml and most preferably 6µg/ml of GroEL may be loaded onto the or each spot.

The array may be incubated in an humidifier at 4°C overnight.

Preferably, from 0.25 to 3 pmole of protein is immobilised on the or each spot. More preferably 0.5 to 2 pmole, more preferably 0.5 to 1.5 pmole and most preferably 1 pmole of protein is immobilised on the or each spot on the array.

BIOLOGICAL SAMPLE DENATURATION

The protein in a biological sample for use in the present invention may be denatured using reagents selected from the group consisting of chaotropic agents, detergents, heat, reducing agents, oxidising agents, laser-induced denaturation and sonication.

Preferably, the chaotropic agent may be selected from guanidine hydrochloride, guanidine thiocyanate, urea, thiourea, sodium thiocyanate and ammonium sulphate

Preferably the detergent may be, for example, sodium dodecyl sulphate.

Preferably, the reducing agent may be selected from dithiothreitol (Cleland's reagent), dithioerythritol and 2-mercaptoethanol.

Preferably, the oxidising agent may be hydrogen peroxide.

The biological sample may be denatured by a combination of the above-mentioned denaturing agents. Most preferably the biological sample is denatured by, for example, a buffer comprising a chelating agent, for example, EDTA; dithiotreitol and guanidine hydrochloride.

The biological sample is denatured for about 1 to 2 hours in the denaturation buffer. The biological sample may be subsequently diluted in binding buffer. This step allows the denatured protein to partially renature and promote the binding of the protein to GroEL. At high salt concentrations, for example, above 5M guanidine HCl, the chaotropic effect of the salt is too great for GroEL to bind to the denatured protein.

ISOLATION OF DENATURED PROTEINS IN A BIOLOGICAL SAMPLE

Referring to Figure 10, the mechanism of protein separation by GroEL is illustrated. GroEL is shown conjugated onto a NeutrAvidin bead. The beads would typically be located in a column. The GroEL may first be primed with ATP (T = ATP). The denatured substrate from the biological sample may be introduced into the column, after being partially renatured, for binding to GroEL. The renatured substrate may interact with the cis cavity of GroEL. The protein may then be encapsulated. In the absence of GroES, GroEL can partially encapsulate proteins to 82kDa or greater (Chaudhuri *et al.*,

2001). After washing to remove proteins that have bound non-specifically, the captured protein(s) are released by the addition ATP in the presence of co-factors Mg^{2+} and K^{+}. When protein separation is carried out in the presence of GroES the protein is limited to ~ 57 kD and below.

The protein separation device according to the invention may be incubated with a biological sample containing denatured proteins. The biological sample is preferably suspended in a physiological buffer. The biological sample may be incubated with the protein separation device for between 1 to 5 hours and most preferably for about 4 hours at ambient room temperature. Alternatively, the incubation period is from 10 to 20 minutes.

Any non-specific binding can be removed by washing the protein separation device in a suitable buffer. The captured proteins of interest may be released by ATP in the presence of Mg^{2+} and K^{+}.

The protein separation device may be processed using, for example, using a suitable output device available from, for example, Ciphergen®.

USES

The protein separation device in accordance with the present invention may be useful in the identification of biological markers for disease. For example, the protein separation device may isolate proteins from a patient suffering from a particular disease that are only expressed in the diseased state when referenced with a normal subject. Alternatively, these isolated proteins may be under-expressed or over-expressed in the diseased state.

The protein separation device may be useful to test the protein composition of a biological sample, with particular reference to biological markers, prior to and subsequent of an administration of a pharmaceutical or neutraceutical compound. The results could give an indication, for example, of the side effects of a particular compound. This may find application in the screening of pharmaceutical or neutraceutical compounds.

The protein separation device may also be useful in the prognosis of a disease state. The protein separation device may be used to screen for biological markers in a biological sample prior, during and after treatment of a disease state to assess the efficacy of a particular treatment regime or protocol.

The protein separation device may further be used to diagnose a disease by assaying for changes in the relative concentrations of important biological markers in a biological sample.

Advantageously, the present invention may find particular application in the diagnosis of disease associated with proteins that have not folded correctly, for example those diseases selected from cystic fibrosis, Alzheimer's disease, emphysema, Huntington's disease, spinocerebellar ataxia type 3, primary lateral sclerosis and amyotrophic lateral sclerosis. Most preferably the present invention may be used in the diagnosis of transmissible spongiform encephalopathies, for example, Creutzfeld-Jakob Disease, variant Creutzfeld-Jakob Disease, Gerstmann-Straussler-Scheinker Syndrome, Fatal Familial Insomnia, Kuru, Atypical Prion Disease, Bovine Spongiform Encephalopathy, Scrapie, Feline Spongiform Encephalopathy, Transmissible Mink

Encephalopathy, Chronic Wasting Disease, Exotic Ungulate Encephalopathy.

The protein separation device may further be used to isolate proteins from any denatured biological sample.

The following example is offered by way of illustration and not by way of limitation.

EXAMPLE 1

This example describes the purification of a modified GroEL protein.

CELL CULTURE

Approximately 6 litres of an *E. coli* bacterial cell culture transformed with an expression plasmid comprising GroEL was incubated in Luria broth (Invitrogen) in a shaker (250 rpm) at 37° C. When the optical density of the culture reached 0.6, 1mM of IPTG (isopropyl-d-thiogalactopyranoside) was added to the culture. The culture was then incubated for a further 4-5 hours. Bacterial cells were subsequently harvested by centrifugation at 4000 x g for 10 minutes and the cell pellets were stored at -80°C.

PURIFICATION OF GROEL

A frozen cell pellet was resuspended in 45 ml of buffer A [50 mM Tris-HCL pH 7.5, 1mM DTT, 0.1mM PMSF (phenylmethylsulfonyl fluoride) and 1mM EDTA]. The cell suspension was passed through a French press three times with an internal cell pressure of 1,000 psi to obtain a cell lysate. The cell lysate was centrifuged at 20,000 x g for 0.5 hour. The supernatant was isolated and supplemented with 20% ammonia sulphate. The supernatent was then injected

into a butyl-toyopearl hydrophobic interaction column (Tosoh Corporation, Japan). The column was pre-equilibrated with 23% ammonia sulphate and 20% methanol in buffer A. The injected sample was allowed to reach equilibrium for 15 minutes. The column was then washed with the buffer A. Subsequently, GroEL was eluted by means of a reverse linear gradient of ammonia sulphate, i.e. a gradient of 23% to 0%, in buffer A.

Eluted fractions containing GroEL were pooled and GroEL was reconstituted into its double heptameric ring configuration by precipitating the pooled fractions in 70% Ammonia sulphate supplemented with 5mM $MgCl_2$ and 3mM ATP. The precipitate was pelleted at 20,000 x g and resuspended in buffer A containing 10% glycerol and stored at -80°C.

Typically 6 litres of cell culture will yield approximately 250 mg of > 95% pure GroEL, see Figure 5 and 6. Figure 5 shows a coomassie-stained SDS-PAGE gel of fractions from a butyl-toyopearl hydrophobic interaction column. GroEL was purified to ~ >95% purity. Lane 1 = Molecular weight marker (SeeBlue Pre-stained Standard, from Invitrogen Catalog code: LC5625) lanes 2-8 are, respectively, 2,4,6,8,10,14,16 µg of total loaded protein. Figure 6 shows a coomassie-stained native PAGE gel showing the purity of reconstituted GroEL-490-14. Lanes 1 and 2 represent 10 and 5 µg of native GroEL-490 respectively. Lane 3 is a molecular weight marker (High molecular weight markers for native electrophoresis from Amersham biosciences ,product code: 17-0445-01).

GroEL-490 was then conjugated onto NeutrAvidin beads and tested for ATPase activity and protein folding properties. The reconstituted GroEL was to function as an ATPase and to be capable of protein folding, see Figure 7 and Figure 8.

Figure 7 demonstrates the ATPase activity of GroEL490 on NeutrAvidin beads using an Enzchek phosphate assay kit from molecular Probes (Catalog code: E-6646). Each data point is the average of two separate experiments and ~ 2 mg of GroEL-490 was used for the activity assay. Figure 8 shows the substrate folding ability of GroEL-490 on NeutrAvidin beads.

The substrate folding ability was tested by the ability to refold denatured mitochondrial malate dehydrogenase (mMDH). The assay protocol was taken from Staniforth *et al.*, 1994. Each data point is the repeat of two separate experiments. Briefly, native mMDH will have the ability to convert NADH (OD at 340nm) to NAD^+ (no OD at 340nm) in the presence of oxaloacetate. Therefore, positive GroEL-490 activity was confirmed by a reduction in OD at 340nm with respect to the control.

PURIFICATION OF GROES

GroES was purified using a DEAE anion exchange column (Biorad). *E. coli* transformed with GroES expression plasmids were grown in a similar way as *E. coli* transformed with GroEL expression plasmids. However, the cell lysate obtained after French pressing was incubated at 80°C for 20 minutes to coagulate heat sensitive proteins present in the lysate. The coagulated proteins were then centrifuged at 12,000 x g for 20 minutes. The supernatant was decanted and injected into DEAE column equilibrated with buffer A. The supernatent was allowed to equilibrate with the column for 20 minutes. The column was then washed with buffer A and GroES was eluted using a linear gradient of 0 to 0.6 M NaCl in buffer A.

Eluted fractions containing GroES were identified by SDS-PAGE, pooled and reconstituted into its heptameric

configuration in 70% ammonia sulphate in the absence of $MgCl_2$ and ATP. Typically, 6 litres of bacterial cell culture will give approximately 150mg of > 95% pure GroES, see Figure 5.

Figure 5 shows a coomassie-stained gel of GroES containing fractions eluted from a column. These fractions were confirmed by SDS-PAGE to be > 95% pure. Lane 1 = molecular weight markers, lanes 2-10 are consecutive fractions from a column (5 ml each).

EXAMPLE 2

This example describes the isolation of proteins from a biological sample using the protein separation device in accordance with the present invention.

Serum was derived from a human blood sample. The Protein separation device comprised GroEL immobilised on a bio-chip array such as those available from Ciphergen® or GroEL conjugated to beads, for example NeutrAvidin beads, available from Pierce. All other reagents were obtained from Sigma.

The protein separation device was prepared as follows:

Approximately 75mg-100mg of GroEL490 was passed through a PD10 (Amersham) desalting column in PBS-EDTA (5mM) buffer. Desalted GroEL490 was biotinylated in PBS-EDTA buffer containing biotin-HPDP based on manufacturer's guidelines for recommended usage. The biotinylation reaction proceeded to completion in ~2 hours. This was confirmed using a spectrometer at an absorbance of 343 nM.

Excess biotin was removed by using a PD10 desalting column. Biotinylated GroEL-490 was then conjugated onto NeutrAvidin beads by incubating the protein with the beads for 1 hour in

PBS buffer (pH 7.5). 10 ml of the NeutrAvidin medium is sufficient to conjugate ~75-100 mg of GroEL-490.

Beads conjugated with GroEL-490 were then packed into a column. The beads were washed with 5 column volumes of buffer W (50 mM Tris-HC, pH 7.5, 100mM KCL, 5mM $MgCl_2$, 0.1 mM DTT, 0.3 mM EDTA). Following this washing step, the column was washed with 2 column volumes of buffer W supplemented with 3 mM ATP to remove any bound endogenous proteins from *E. coli*. The column was then washed with 8 column volumes of buffer W. The column was then ready for use.

75µl of human serum was denatured using 25µl of denaturation buffer (6M guanidine-HCl, 2mM EDTA and 10mM dithiothreitol) at ambient room temperature for 1 hour.

The serum was then diluted 30-50 fold in binding buffer (50mM Tris-HCl, pH 7.4; 10mM $MgCl_2$; 10mM KCl) and immediately loaded on to the column. The serum was left on the column for 20 minutes. The column was then washed with 10 column volumes of buffer W.

At the end of this wash step proteins bound to GroEL-490, regardless of their state of folding, eluted from the column with 3 column volumes of buffer W supplemented with 3mM ATP. Eluted fractions were collected and protein peaks were determined by BCA assay (invitrogen). Eluted fractions of interest were pooled and proteins were concentrated for identification using Liquid Chromatography Mass Spectroscopy.

The data obtained showed that by means of comparison as based on LC-MS-MS results, the protein separation device of the present invention can generate more information (more than seven-fold) from a biological sample (see Table 1) with

respect to a conventional method of enriching low molecular weight proteins, for example, gel filtration (See Table 2). Also the distribution of capture protein species is more even than those by gel filtration (Table 1 and Table 2).

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

DTASelect v1.9 Normal serum GroEL capture

Table 1

Locus	Sequence Count	Spectrum Count	Sequence Coverage	MolWt	pI	Descriptive Name
Unique	FileName	XCorr	DeltCN	CalcM+H+	TotalIntensity	SpScore IonProportion Redundancy Sequence
gi|4502027|ref|NP_000	89	475	77.20%	69367	6.3	albumin precursor; PRO0883 protein [Homo sapiens]
gi|10442822|ref|NP_05	3	113	1.40%	527615	6	baculoviral IAP repeat-containing 6 [Homo sapiens]
gi|42659147|ref|XP_37	3	110	11.80%	50328	5.7	similar to CXYorf1-related protein [Homo sapiens]
gi|4557871|ref|NP_001	36	60	47.70%	77050	7.1	transferrin; PRO2086 protein [Homo sapiens]
gi|20143922|ref|NP_59	48	56	3.80%	3013989	6.7	titin isoform novex-2; connectin; CMH9, included; cardiomyopathy, dilated 1G (autosomal dominant) [Homo sapiens]
gi|20143967|ref|NP_61	1	53	1.60%	110059	8.5	kinesin family member 23 isoform 1; mitotic kinesin-like 1; kinesin-like 5 (mitotic kinesin-like protein 1) [Homo sapiens]
gi|4557225|ref|NP_000	31	43	28.10%	163277	6.4	alpha 2 macroglobulin precursor [Homo sapiens]
gi|4557385|ref|NP_000	28	43	24.80%	187163	6.4	complement component 3 precursor; acylation-stimulating protein cleavage product [Homo sapiens]
gi|4504253|ref|NP_002	3	41	23.10%	15145	10.7	H2A histone family, member X; H2AX histone [Homo sapiens]
gi|9966821|ref|NP_065	2	41	6.60%	68960	7.6	ectonucleoside triphosphate diphosphohydrolase 7; lysosomal apyrase-like protein 1 [Homo sapiens]
gi|4505723|ref|NP_002	2	38	6.50%	44130	8.1	peroxisome biogenesis factor 13 [Homo sapiens]
gi|11321561|ref|NP_00	19	35	37.20%	51676	7	hemopexin [Homo sapiens]
gi|34485727|ref|NP_00	2	35	1.20%	128153	6.9	hematopoietic protein 1; membrane-associated protein hem-1 [Homo sapiens]
gi|21361612|ref|NP_07	2	34	4.30%	61949	8	HpaII tiny fragments locus 9C isoform2 [Homo sapiens]
gi|12083581|ref|NP_05	4	34	8.10%	138567	6.2	phosphoinositide-specific phospholipase C beta 1 isoform a; 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta 1; PLC-beta-1; triphosphoinositide phosphodiest
gi|37540833|ref|XP_03	3	33	1.30%	341363	7.1	MAX dimerization protein 5 [Homo sapiens]
gi|18254460|ref|NP_54	1	30	14.30%	30179	9.3	SPRY domain-containing SOCS box protein SSB-4 [Homo sapiens]
gi|4885637|ref|NP_005	3	30	14.40%	53818	4.7	target of myb1; target of myb 1; target of myb1 (chicken) homolog [Homo sapiens]
gi|14149734|ref|NP_06	3	28	12.70%	54235	5.9	coronin, actin binding protein, 1B [Homo sapiens]
gi|42661355|ref|XP_29	4	28	4.20%	75410	5.8	similar to KIAA1074 protein [Homo sapiens]
gi|21361452|ref|NP_05	4	27	10.90%	73427	7.8	glutaminase C; L-glutamine amidohydrolase; K-glutaminase; glutaminase, phosphate-activated [Homo sapiens]
gi|4507825|ref|NP_001	1	26	4.20%	60695	8.3	UDP glycosyltransferase 2 family, polypeptide B7; UDP-glucuronyltransferase, family 2, beta-7 [Homo sapiens]
gi|40805845|ref|NP_00	5	24	3.90%	305244	7.8	DNA polymerase theta isoform 1; polymerase (DNA-directed), theta [Homo sapiens]
gi|20149635|ref|NP_05	2	22	7.80%	40573	5.1	p47 protein isoform a [Homo sapiens]
gi|42718017|ref|NP_97	2	22	2.50%	98434	6.6	retinoblastoma binding protein 8 isoform b; CTBP-interacting protein; retinoblastoma-interacting myosin-like [Homo sapiens]
gi|27659724|ref|NP_00	2	21	12.10%	25173	8.5	synovial sarcoma, X breakpoint 2 isoform a; sarcoma, synovial, X-chromosome-related 2; SSX2 protein [Homo sapiens]
gi|15208665|ref|NP_15	2	21	10.40%	49773	7.9	tripartite motif protein TRIM14 isoform alpha; tripartite motif protein TRIM14; tripartite motif protein 14 [Homo sapiens]
gi|21361198|ref|NP_00	10	20	23.20%	46723	5.6	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1; Protease inhibitor (alpha-1-antitrypsin); protease inhibitor 1 (anti-e
gi|41151008|ref|XP_09	3	19	6.40%	79360	9.2	similar to BC331191_1 [Homo sapiens]
gi|41203848|ref|XP_37	6	19	12.00%	84926	4.8	similar to Ig alpha-2 chain C region [Homo sapiens]
gi|4557485|ref|NP_000	13	19	20.90%	122205	5.7	ceruloplasmin (ferroxidase); Ceruloplasmin [Homo sapiens]
gi|4502153|ref|NP_000	16	19	6.70%	515569	7.1	apolipoprotein B precursor; apoB-100; apoB-48 [Homo sapiens]
gi|22094125|ref|NP_06	2	18	4.40%	98705	9.3	zinc finger protein 28; zinc finger factor X6 [Homo sapiens]
gi|41151031|ref|XP_37	1	17	39.80%	10834	11.1	hypothetical protein XP_378771 [Homo sapiens]

gi|4503555|ref|NP_001	2	17	6.30%	70730	5.6 E74-like factor 4 (ets domain transcription factor) [Homo sapiens]
gi|32484977|ref|NP_05	1	17	2.20%	85835	7.3 angiomotin like 2; Leman coiled-coil protein; angiomotin-like protein 2 [Homo sapiens]
gi|22044446|ref|XP_08	1	16	4.50%	41376	5.8 similar to zinc finger, CW-type with PWWP domain 1 [Homo sapiens]
gi|17318569|ref|NP_00	13	16	27.20%	66067	8.1 keratin 1; Keratin-1; cytokeratin 1; hair alpha protein [Homo sapiens]
gi|38569480|ref|NP_05	1	16	1.50%	114119	6 myocardin-related transcription factor B; megakaryoblastic leukemia 2 [Homo sapiens]
gi|22779934|ref|NP_07	5	16	8.20%	151581	6.4 WD repeat membrane protein PWDMP [Homo sapiens]
gi|19747267|ref|NP_59	10	16	0.50%	3816051	6.3 titin isoform N2-A; connectin; CMH9, included; cardiomyopathy, dilated 1G (autosomal dominant) [Homo sapiens]
gi|7706310|ref|NP_057	2	15	8.70%	47620	10 LUC7-like 2; CGI-74 protein; CGI-59 protein; H_NH0792N18.3 [Homo sapiens]
gi|42656543|ref|XP_37	12	15	4.70%	722938	6.5 hypothetical protein XP_376158 [Homo sapiens]
gi|42661301|ref|XP_37	1	14	13.10%	16384	8.7 hypothetical protein XP_378639 [Homo sapiens]
gi|4758148|ref|NP_004	1	14	5.10%	36522	4.8 DNA fragmentation factor, 45 kD, alpha polypeptide; DNA fragmentation factor, 45 kD, alpha subunit; DFF45 [Homo sapiens]
gi|4502503|ref|NP_000	8	14	12.10%	67033	7.3 complement component 4 binding protein, alpha; Complement component 4-binding protein, alpha polypeptide; complement component 4-binding protein, alpha [Homo sapiens]
gi|29745114|ref|XP_29	1	14	1.10%	75996	10.2 dendrin [Homo sapiens]
gi|19923374|ref|NP_00	6	14	4.80%	274881	6.9 human immunodeficiency virus type I enhancer binding protein 2; human immunodeficiency virus type I enhancer-binding protein 2 [Homo sapiens]
gi|41146759|ref|XP_37	3	14	2.30%	313978	6.8 odd Oz/Ten-m homolog 3 [Homo sapiens]
gi|45439359|ref|NP_00	6	14	4.90%	346901	6.4 triple functional domain (PTPRF interacting) [Homo sapiens]
gi|19882213|ref|NP_11	12	14	4.60%	692686	4.6 very large G-protein coupled receptor 1; very large G protein-coupled receptor 1; G protein-coupled receptor 98 [Homo sapiens]
gi|41150939|ref|XP_37	1	13	19.80%	10777	9.3 similar to Nonhistone chromosomal protein HMG-14 (High-mobility group nucleosome binding domain 1) [Homo sapiens]
gi|38569396|ref|NP_00	1	13	10.30%	24860	9.7 insulin-like 6 precursor; relaxin/insulin-like factor 1; insulin-like peptide 5 [Homo sapiens]
gi|4557321|ref|NP_000	10	13	31.10%	30778	5.8 apolipoprotein A-I precursor [Homo sapiens]
gi|11276085|ref|NP_06	1	13	4.50%	59941	7.9 UDP glycosyltransferase 1 family, polypeptide A9 [Homo sapiens]
gi|4505695|ref|NP_002	3	13	8.60%	63152	7.4 3-phosphoinositide dependent protein kinase-1; PkB kinase [Homo sapiens]
gi|4502193|ref|NP_001	2	13	7.10%	67585	9 v-raf murine sarcoma 3611 viral oncogene homolog 1; Oncogene ARAF1 [Homo sapiens]
gi|29731965|ref|XP_29	7	13	14.60%	161753	5.6 KIAA0804 protein [Homo sapiens]
gi|5803088|ref|NP_005	6	13	7.60%	181551	6.3 MAP/ERK kinase kinase 4 isoform a; SSK2/SSK22 MAP kinase kinase kinase, yeast, homolog of [Homo sapiens]
gi|24308217|ref|NP_06	8	13	10.20%	201300	7.6 KIAA1330 protein [Homo sapiens]
gi|42658790|ref|XP_37	6	13	6.90%	232552	6.8 similar to hypothetical protein [Homo sapiens]
gi|37541569|ref|XP_04	11	13	6.50%	408536	7.7 zinc finger protein 469 [Homo sapiens]
gi|24431966|ref|NP_00	2	12	9.30%	51988	5.8 stress 70 protein chaperone, microsome-associated, 60kDa; Stress 70 protein chaperone, microsome-associated, p60; human microsomal stress 70 protein ATPase core [Homo sap
gi|42741679|ref|NP_03	2	12	4.10%	98082	6.6 ATPase, H+ transporting, lysosomal V0 subunit a isoform 2 [Homo sapiens]
gi|21361551|ref|NP_00	4	12	10.60%	137009	6.3 solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1) [Homo sapiens]
gi|4504375|ref|NP_000	10	12	9.10%	139125	6.7 H factor 1 (complement); H factor-1 (complement); complement factor H; factor H-like 1 [Homo sapiens]
gi|4502221|ref|NP_001	1	12	1.60%	171568	6.6 Rho GTPase activating protein 5; RhoGAP5; p190-B [Homo sapiens]
gi|41146538|ref|XP_29	3	12	3.20%	235179	7.3 similar to KIAA2018 protein [Homo sapiens]
gi|4508019|ref|NP_003	8	12	3.30%	416471	7.5 bassoon; zinc finger protein 231; neuronal double zinc finger protein [Homo sapiens]
gi|42661056|ref|XP_29	2	11	5.20%	64976	8 similar to ataxin 2 binding protein 1 isoform gamma; hexaribonucleotide binding protein 1 [Homo sapiens]

gi|42476164|ref|NP_00	1	11	3.80%	76472	6.8 POU domain, class 2, transcription factor 1; Octamer-binding transcription factor-1 [Homo sapiens]
gi|4502175|ref|NP_001	2	11	2.40%	176409	7.1 apical protein of Xenopus-like; APX homolog of Xenopus [Homo sapiens]
gi|4502501|ref|NP_000	6	11	4.90%	192796	7.3 complement component 4B proprotein [Homo sapiens]
gi|19718755|ref|NP_00	2	11	2.50%	198839	6.4 RAN-binding protein 2-like 1 isoform 1; sperm membrane protein BS-63; RAN-binding protein 2-like 1 [Homo sapiens]
gi|5453571|ref|NP_006	5	11	6.10%	206708	5.9 brefeldin A-inhibited guanine nucleotide-exchange protein 1 [Homo sapiens]
gi|20977541|ref|NP_06	5	11	5.20%	217325	7.8 Rho GTPase activating protein 21; Rho-GTPase activating protein 10 [Homo sapiens]
gi|33636748|ref|NP_07	5	11	4.60%	278806	6 hypothetical protein FLJ21439 [Homo sapiens]
gi|41150991|ref|XP_37	3	11	1.70%	305700	8.2 NYD-SP11 protein [Homo sapiens]
gi|21362014|ref|NP_07	1	10	3.50%	79353	7.2 hypothetical protein FLJ22344 [Homo sapiens]
gi|42734315|ref|NP_05	3	10	3.40%	90395	7.7 KIAA0317 [Homo sapiens]
gi|17978502|ref|NP_00	7	10	18.90%	91819	8.7 alpha 1 type IX collagen isoform 1 precursor; collagen IX, alpha-1 polypeptide; cartilage-specific short collagen [Homo sapiens]
gi|4504783|ref|NP_002	8	10	16.60%	106714	7 inter-alpha (globulin) inhibitor, H2 polypeptide [Homo sapiens]
gi|18201915|ref|NP_54	9	10	16.90%	159976	8.6 alpha 2 type XI collagen isoform 3 preproprotein [Homo sapiens]
gi|41204884|ref|XP_03	7	10	4.30%	396079	5.7 START domain containing 9 [Homo sapiens]
gi|42659582|ref|XP_37	9	10	5.50%	485019	6.8 dynein, cytoplasmic, heavy polypeptide 2 [Homo sapiens]
gi|31083306|ref|NP_11	9	10	3.80%	613524	6.5 hemicentin; fibulin 6 [Homo sapiens]
gi|33188445|ref|NP_03	5	10	1.80%	620426	5.4 microfilament and actin filament cross-linker protein isoform a; 620 kDa actin binding protein; actin cross-linking factor; macrophin 1; trabeculin-alpha; actin cross-lin
gi|6912406|ref|NP_036	1	9	6.60%	32542	5.9 3-hydroxyanthranilate 3,4-dioxygenase [Homo sapiens]
gi|20127446|ref|NP_00	2	9	4.40%	88054	6.1 integrin, beta 5 [Homo sapiens]
gi|32526894|ref|NP_84	3	9	8.50%	92537	6.7 hypothetical protein FLJ35834 [Homo sapiens]
gi|39930403|ref|NP_06	2	9	8.10%	99267	6 leucine-rich repeat-containing G protein-coupled receptor 6 [Homo sapiens]
gi|31543297|ref|NP_00	2	9	5.00%	104372	6.2 neutral sphingomyelinase (N-SMase) activation associated factor [Homo sapiens]
gi|4885643|ref|NP_005	4	9	7.30%	111431	6 tumor protein p53 binding protein, 2; apoptosis-stimulating protein of p53, 2 [Homo sapiens]
gi|19923493|ref|NP_06	6	9	10.50%	127464	7.5 phosphoinositol 3-phosphate-binding protein-2 [Homo sapiens]
gi|24850456|ref|NP_05	2	9	4.30%	128820	8.4 SMC5 protein [Homo sapiens]
gi|7706513|ref|NP_057	1	9	1.60%	137136	6.3 PDZ domain-containing guanine nucleotide exchange factor I; rap guanine nucleotide exchange factor; PDZ domain containing guanine nucleotide exchange factor (GEF) 2 [Homo
gi|4885399|ref|NP_005	4	9	5.60%	141541	7.2 chondroitin sulfate proteoglycan 6 (bamacan); human chromosome-associated polypeptide (bamacan); SMC3 structural maintenance of chromosomes 3-like 1 (yeast) [Homo sapiens]
gi|42659545|ref|XP_37	6	9	6.40%	196409	5.7 ankyrin repeat domain 26 [Homo sapiens]
gi|39652624|ref|NP_03	9	9	10.60%	233152	7.4 extra spindle poles like 1 [Homo sapiens]
gi|38202219|ref|NP_07	2	9	1.50%	240001	6.7 hypothetical protein FLJ12178 [Homo sapiens]

DTASelect v1.9

Normal serum Gel Filtration

Table 2

Locus	Sequence Count	Spectrum Count	Sequence Coverage	MolWt	pI	Descriptive Name
gi|4502027|ref|NP_000	109	1439	81.10%	69367	6.3	albumin precursor; PRO0883 protein [Homo sapiens]
gi|4557385|ref|NP_000	62	90	47.10%	187163	6.4	complement component 3 precursor; acylation-stimulating protein cleavage product [Homo sapiens]
gi|4557225|ref|NP_000	55	85	48.80%	163277	6.4	alpha 2 macroglobulin precursor [Homo sapiens]
gi|4557871|ref|NP_001	33	67	56.60%	77050	7.1	transferrin; PRO2086 protein [Homo sapiens]
gi|4557321|ref|NP_000	25	57	46.80%	30778	5.8	apolipoprotein A-I precursor [Homo sapiens]
gi|21361198|ref|NP_00	20	49	40.20%	46723	5.6	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1; Protease inhibitor (alpha-1-antitrypsin); protease inhibitor 1 (anti-e
gi|11321561|ref|NP_00	23	37	49.60%	51676	7	hemopexin [Homo sapiens]
gi|32483410|ref|NP_00	22	35	48.70%	52918	5.4	vitamin D-binding protein precursor; vitamin D-binding alpha-globulin [Homo sapiens]
gi|4502149|ref|NP_001	6	31	43.00%	11175	6.6	apolipoprotein A-II precursor [Homo sapiens]
gi|4502501|ref|NP_000	24	31	21.70%	192796	7.3	complement component 4B proprotein [Homo sapiens]
gi|4826762|ref|NP_005	12	28	44.60%	45205	6.6	haptoglobin [Homo sapiens]
gi|4557485|ref|NP_000	22	26	31.20%	122205	5.7	ceruloplasmin (ferroxidase); Ceruloplasmin [Homo sapiens]
gi|4504253|ref|NP_002	4	23	33.60%	15145	10.7	H2A histone family, member X; H2AX histone [Homo sapiens]
gi|4504255|ref|NP_002	3	22	25.00%	13553	10.6	H2A histone family, member Z; H2AZ histone [Homo sapiens]
gi|31542984|ref|NP_00	16	20	27.80%	103357	7	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein); inter-alpha (globulin) inhibitor, H polypeptide-like 1; Inter-alpha (globulin) inhibitor,
gi|4504783|ref|NP_002	14	20	23.40%	106714	7	inter-alpha (globulin) inhibitor, H2 polypeptide [Homo sapiens]
gi|21264330|ref|NP_54	1	18	2.00%	109909	8.4	skeletrophin; novelzin [Homo sapiens]
gi|4504165|ref|NP_000	11	17	24.80%	85697	6.3	gelsolin isoform a [Homo sapiens]
gi|4502153|ref|NP_000	15	17	5.90%	515569	7.1	apolipoprotein B precursor; apoB-100; apoB-48 [Homo sapiens]
gi|41203848|ref|XP_37	5	15	14.30%	84926	4.8	similar to Ig alpha-2 chain C region [Homo sapiens]
gi|4557327|ref|NP_000	12	14	29.60%	38312	8	beta-2-glycoprotein I precursor [Homo sapiens]
gi|4502151|ref|NP_000	10	14	29.50%	45381	5.4	apolipoprotein A-IV precursor [Homo sapiens]
gi|4502397|ref|NP_001	13	14	20.00%	85505	7	complement factor B preproprotein; C3 proactivator; C3 proaccelerator; glycine-rich beta-glycoprotein; C3/C5 convertase [Homo sapiens]
gi|4502005|ref|NP_001	10	13	41.70%	39325	5.7	alpha-2-HS-glycoprotein; Alpha-2HS-glycoprotein [Homo sapiens]
gi|41150283|ref|XP_05	2	13	3.50%	146662	5.3	KIAA1005 protein [Homo sapiens]
gi|9257232|ref|NP_000	9	12	32.30%	23512	5	orosomucoid 1 precursor; Orosomucoid-1 (alpha-1-acid glycoprotein-1); alpha-1-acid glycoprotein 1 [Homo sapiens]
gi|4505881|ref|NP_000	11	12	27.90%	90569	7.2	plasminogen [Homo sapiens]
gi|21071030|ref|NP_57	9	11	32.10%	54254	5.9	alpha 1B-glycoprotein [Homo sapiens]
gi|4503635|ref|NP_000	9	11	19.10%	70037	5.9	coagulation factor II precursor; prothrombin [Homo sapiens]
gi|4504375|ref|NP_000	10	11	12.30%	139125	6.7	H factor 1 (complement); H factor-1 (complement); complement factor H; factor H-like 1 [Homo sapiens]
gi|4503481|ref|NP_001	3	10	5.70%	50119	6.7	eukaryotic translation elongation factor 1 gamma; elongation factor 1-gamma; EF-1-gamma; eEF-1B gamma; translation elongation factor eEF-1 gamma chain; PRO1608; pancreatic
gi|18201911|ref|NP_00	4	10	10.90%	54336	5.8	vitronectin precursor; serum spreading factor; somatomedin B; complement S-protein; epibolin [Homo sapiens]
gi|4504781|ref|NP_002	10	10	22.20%	101402	6.8	inter-alpha (globulin) inhibitor, H1 polypeptide [Homo sapiens]
gi|4504289|ref|NP_003	1	9	23.50%	15404	11.1	H3 histone family, member F [Homo sapiens]
gi|5174411|ref|NP_005	8	9	32.00%	38088	5.5	CD5 antigen-like (scavenger receptor cysteine rich family); Spalpha [Homo sapiens]
gi|5902134|ref|NP_009	1	9	3.00%	51026	6.7	coronin, actin binding protein, 1A; coronin, actin-binding, 1A; coronin, actin-binding protein, 1A; coronin-1 [Homo sapiens]
gi|29336063|ref|NP_00	1	9	0.70%	206846	6.4	plexin B3; plexin 6; plexin-B3 [Homo sapiens]
gi|4557323|ref|NP_000	5	8	20.20%	10852	5.4	apolipoprotein C-III precursor [Homo sapiens]

gi|4507621|ref|NP_003	1	8	9.90%	21338	8.7 troponin I, skeletal, fast; Troponin I fast twitch 2; Troponin I, fast-twitch skeletal muscle isoform [Homo sapiens]
gi|7669492|ref|NP_002	3	8	22.40%	36053	8.5 glyceraldehyde-3-phosphate dehydrogenase [Homo sapiens]
gi|4758496|ref|NP_004	1	8	7.80%	39489	9.8 H2A histone family, member Y isoform 2; histone macroH2A1.2; histone macroH2A1.1 [Homo sapiens]
gi|4501843|ref|NP_001	6	8	19.60%	48637	6.2 alpha-1-antichymotrypsin, precursor; alpha-1-antichymotrypsin; antichymotrypsin [Homo sapiens]
gi|17318569|ref|NP_00	5	8	14.80%	66067	8.1 keratin 1; Keratin-1; cytokeratin 1; hair alpha protein [Homo sapiens]
gi|32307124|ref|NP_00	1	8	1.50%	154891	7.5 nuclear receptor coactivator 3 isoform b; amplified in breast cancer-1; thyroid hormone receptor activator molecule 1; receptor-associated coactivator 3; steroid receptor
gi|38016947|ref|NP_00	7	8	4.50%	188304	6.5 complement component 5 [Homo sapiens]
gi|10947052|ref|NP_00	3	8	1.10%	433749	5.1 ankyrin 2 isoform 1; ankyrin-2, nonerythrocytic; ankyrin-B; ankyrin, brain; ankyrin, neuronal; ankyrin, nonerythroid; Long QT syndrome-4; long (electrocardiographic) QT s
gi|4505409|ref|NP_002	2	7	7.20%	17298	8.4 nucleoside-diphosphate kinase 2; non-metastatic cells 2, protein (NM23) expressed in; c-myc transcription factor [Homo sapiens]
gi|4507949|ref|NP_003	1	7	8.10%	28082	4.8 tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide; 14-3-3 protein beta/alpha; protein kinase C inhibitor protein-1; protein 1054; br
gi|4504893|ref|NP_000	5	7	16.20%	47883	6.7 kininogen [Homo sapiens]
gi|4502261|ref|NP_000	6	7	13.40%	52602	6.7 serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1; antithrombin III [Homo sapiens]
gi|4502503|ref|NP_000	6	7	14.70%	67033	7.3 complement component 4 binding protein, alpha; Complement component 4-binding protein, alpha polypeptide; complement component 4-binding protein, alpha [Homo sapiens]
gi|4502493|ref|NP_001	5	7	13.60%	80200	6.3 complement component 1, r subcomponent [Homo sapiens]
gi|40254816|ref|NP_00	3	7	7.40%	84674	5 heat shock 90kDa protein 1, alpha; heat shock 90kD protein 1, alpha [Homo sapiens]
gi|4504347|ref|NP_000	6	6	60.60%	15258	8.7 alpha 1 globin [Homo sapiens]
gi|28872725|ref|NP_00	1	6	3.60%	47464	6.5 proteasome 26S non-ATPase subunit 11; 26S proteasome regulatory subunit 9 [Homo sapiens]
gi|4557287|ref|NP_000	4	6	16.30%	53154	6.3 angiotensinogen precursor; angiotensin II precursor; pre-angiotensinogen; angiotensin I [Homo sapiens]
gi|31377715|ref|NP_07	2	6	7.60%	66841	8.3 solute carrier family 13 member 3; sodium-dependent high affinity dicarboxylate transporter 3; Na(+)/dicarboxylate cotransporter 3 [Homo sapiens]
gi|21735575|ref|NP_06	1	6	3.50%	71686	9.3 junctophilin 1; mitsugumin72; junctophilin type1 [Homo sapiens]
gi|5032135|ref|NP_005	2	6	1.20%	174258	7.4 ATP-binding cassette, sub-family C, member 9 isoform SUR2A; sulfonylurea receptor 2A [Homo sapiens]
gi|42661087|ref|XP_08	1	6	0.40%	446640	6.5 FLJ46675 protein [Homo sapiens]
gi|27484057|ref|XP_21	2	5	22.20%	12427	6.6 hypothetical protein XP_211339 [Homo sapiens]
gi|11036646|ref|NP_05	2	5	18.30%	13944	10.4 H2B histone family, member S [Homo sapiens]
gi|42661381|ref|XP_37	1	5	10.90%	19331	8.3 hypothetical protein XP_378743 [Homo sapiens]
gi|5803139|ref|NP_006	5	5	42.20%	22868	5.7 RBP4 gene product [Homo sapiens]
gi|4557325|ref|NP_000	5	5	17.40%	36154	5.7 apolipoprotein E [Homo sapiens]
gi|19923106|ref|NP_00	5	5	22.00%	39731	5.2 paraoxonase 1; Paraoxonase [Homo sapiens]
gi|4502595|ref|NP_001	3	5	6.40%	45141	6 corticosteroid binding globulin precursor; corticosteroid binding globulin; alpha-1 antiproteinase, antitrypsin [Homo sapiens]
gi|4506801|ref|NP_003	1	5	4.10%	70869	4.8 secretogranin II precursor; Chromogranin C (secretogranin II); secretoneurin precursor; EM66 precursor [Homo sapiens]
gi|20149594|ref|NP_03	1	5	3.70%	83264	5 heat shock 90kDa protein 1, beta; heat shock 90kD protein 1, beta; Heat-shock 90kD protein-1, beta [Homo sapiens]
gi|42662523|ref|XP_37	2	5	2.20%	130322	6.8 zinc finger, BED domain containing 4 [Homo sapiens]
gi|15451892|ref|NP_05	1	5	0.80%	138929	5.5 translation initiation factor IF2 [Homo sapiens]
gi|45439327|ref|NP_00	3	5	3.40%	204676	5.6 periplakin; 195 kDa cornified envelope precursor; 190 kDa paraneoplastic pemphigus antigen [Homo sapiens]
gi|20143922|ref|NP_59	4	5	0.30%	3E+06	6.7 titin isoform novex-2; connectin; CMH9, included; cardiomyopathy, dilated 1G (autosomal dominant) [Homo sapiens]
gi|41222847|ref|XP_37	2	4	12.10%	10382	9.5 hypothetical protein XP_378690 [Homo sapiens]

gi|41150939|ref|XP_37	1	4	19.80%	10777	9.3	similar to Nonhistone chromosomal protein HMG-14 (High-mobility group nucleosome binding domain 1) [Homo sapiens]
gi|24638446|ref|NP_00	1	4	22.50%	13988	10.9	H2A histone family, member Q; H2A histone; histone IIa [Homo sapiens]
gi|4507725|ref|NP_000	3	4	35.40%	15887	5.7	transthyretin (prealbumin, amyloidosis type I); Transthyretin (prealbumin) [Homo sapiens]
gi|4504349|ref|NP_000	4	4	38.80%	15998	7.3	beta globin [Homo sapiens]
gi|42659183|ref|XP_37	2	4	14.40%	20014	11.5	hypothetical protein XP_379629 [Homo sapiens]
gi|7705753|ref|NP_057	3	4	20.40%	26017	9.1	complement component 1, q subcomponent, alpha polypeptide precursor; complement component C1q, A chain [Homo sapiens]
gi|12056465|ref|NP_00	1	4	5.90%	33784	10.2	fibrillarin; 34-kD nucleolar scleroderma antigen; RNA, U3 small nucleolar interacting protein 1 [Homo sapiens]
gi|4502337|ref|NP_001	4	4	18.80%	34259	6.1	alpha-2-glycoprotein 1, zinc; Alpha-2-glycoprotein, zinc [Homo sapiens]
gi|11321587|ref|NP_00	3	4	30.60%	37662	7.6	H factor (complement)-like 1 [Homo sapiens]
gi|4885049|ref|NP_005	2	4	8.20%	42019	5.4	actin, alpha, cardiac muscle precursor [Homo sapiens]
gi|4503645|ref|NP_000	2	4	11.20%	51594	7.2	coagulation factor VII precursor isoform a; Coagulation factor VII; eptacog alfa [Homo sapiens]
gi|39930485|ref|NP_44	1	4	3.40%	52689	8.4	mitochondrial Ca2+-dependent solute carrier [Homo sapiens]
gi|4504489|ref|NP_000	4	4	13.70%	59578	7.5	histidine-rich glycoprotein precursor; histidine-proline rich glycoprotein; thrombophilia due to elevated HRG, included [Homo sapiens]
gi|4502511|ref|NP_001	4	4	11.40%	63173	5.6	complement component 9 [Homo sapiens]
gi|4503629|ref|NP_000	3	4	12.40%	67818	7.7	coagulation factor XII precursor; Hageman factor [Homo sapiens]
gi|21361845|ref|NP_44	4	4	15.50%	67970	7.7	peptidoglycan recognition protein L precursor [Homo sapiens]
gi|4507429|ref|NP_003	2	4	5.10%	73629	8.4	tec protein tyrosine kinase [Homo sapiens]
gi|4502495|ref|NP_001	3	4	6.00%	76685	5	complement component 1, s subcomponent [Homo sapiens]
gi|13129040|ref|NP_07	2	4	6.60%	80682	8	spermatogenesis associated 5-like 1 [Homo sapiens]
gi|17978489|ref|NP_00	3	4	5.80%	81743	7.1	CD97 antigen isoform 2 precursor; leukocyte antigen CD97; seven-span transmembrane protein [Homo sapiens]
gi|31377758|ref|NP_06	2	4	7.60%	99444	4.6	aftiphilin protein isoform 1 [Homo sapiens]
gi|14149661|ref|NP_05	1	4	2.20%	108793	6.6	Rab6-interacting protein 2 isoform alpha [Homo sapiens]
gi|40805104|ref|NP_00	1	4	2.50%	119198	9.5	topoisomerase I binding, arginine/serine-rich; tumor protein p53-binding protein; topoisomerase I binding protein [Homo sapiens]
gi|40786422|ref|NP_95	3	4	6.10%	148944	4.9	dachsous-like [Homo sapiens]
gi|4504949|ref|NP_002	1	4	0.70%	201882	6.3	laminin, alpha 4 precursor [Homo sapiens]
gi|28872786|ref|NP_06	1	4	0.80%	215035	5.6	CDK5 regulatory subunit associated protein 2; CDK5 activator-binding protein C48 [Homo sapiens]